

## INFRARED THERMOGRAPHY

The present application claims priority from United States Provisional Application No. 60/085,736, filed May 15, 1998, the entire disclosure of which is incorporated herein by reference.

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### TECHNICAL FIELD

The present invention relates, in general, to thermography and, in particular, to a method of using infrared thermography to monitor physiological and molecular events that elicit a thermogenic response in animals (including humans), plants, tissues, cells and cell-free systems. The present method can be used for screening, identifying, and ranking drug candidates for multiple diseases, disorders and conditions.

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### BACKGROUND

Thermodynamics is a science concerned with relations between work and heat. Virtually every chemical reaction or physiological process in animals or cells occurs with the absorption or generation of heat and thus, any heat absorbed or generated by a system is proportional to the amount of work done. Consequently, measurement of heat output (*i.e.*, thermogenesis) can be used to estimate the energy used in or produced by chemical reactions and physiological processes.

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Various methods (*e.g.*, Northern or Western-blotting) are available for detecting the expression of proteins that regulate thermogenesis in cells (*e.g.*, uncoupling proteins, UCPs), but these methods are labor

intensive and do not directly measure protein activity. Guanosine 5'-diphosphate (GDP)-binding assays and fluorescent dyes (e.g., JC-1 or rhodamine derivatives) provide a direct measure of UCP activity (Nedergaard and Cannon, Am. J. Physiol. 248(3 Pt 1):C365-C371 (1985); (Reers et al, Biochemistry 30:4480-4486 (1991)). However, GDP-binding assays require protein purification and use of dyes is limited because of non-selective staining, cytotoxicity, and metabolism of the dyes by cells. More importantly, all of these techniques fail to directly measure real-time fluctuations in thermogenesis and are invasive.

10 Bomb calorimeters and microcalorimeters provide a means for quantitatively measuring the heat generated or consumed by cultured cells (Bottcher and Furst, J. Biochem. Biophys. Methods 32: 191-194 (1996)) or chemical reactions. However, despite recent progress in developing multichannel calorimeters, methods for rapidly analyzing  
15 changes in heat in multiple simultaneous reactions ( $\geq 60$ ) are not available. Moreover, temperature gradations over fixed surface areas, such as those in cell culture plates or on the surface of skin, cannot be measured using calorimeters.

Infrared thermometers have been developed that can measure the  
20 magnitude of infrared energy emitted from a specific body site (e.g., the ear canal). These instruments, however, cannot be used to measure heat production of isolated cells, tissues, or chemical reactions and cannot provide real time measurements of heat output by multiple samples over extended periods of time. Moreover, these devices do not provide images  
25 over large surface areas.

Infrared interactance instruments have also been developed. Unlike infrared thermometers, these instruments contain diodes that emit near radiation at wavelengths of <1000 nm. Since these instruments measure the absorption of near infrared radiation, they do not provide an accurate measure of thermogenesis.

The present invention provides a rapid non-invasive method of measuring real-time thermogenesis in animals, plants, tissues and isolated cells, including cells in culture. This invention extends to molecular interactions, such as receptor-ligand binding, enzyme catalysis, and other chemical reactions that alter heat output. The present method, which is based on the use of infrared thermography, can be used to screen and identify drug candidates for treating various diseases, disorders and conditions.

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### SUMMARY OF THE INVENTION

The present invention relates generally to a method of monitoring physiological changes and molecular interactions using infrared thermography. Infrared thermography provides a non-invasive approach to analyze the effects of any of a variety of agents on heat production in animals, plants, cells in culture, and chemical reactions in cell-free systems. The invention makes it possible to screen compounds for their ability to alter heat dissipation, and to identify compounds that have application in the treatment of various diseases, disorder and conditions.

Objects and advantages of the present invention will be clear from the description that follows.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic of an apparatus suitable for use in imaging infrared thermogenesis in cells in culture. 1=Infrared camera; 2=cell culture incubator ( $37 \pm .02^\circ\text{C}$ ); 3=isothermal chamber; 4=plate holder; 5=computer interface.

Figure 2. Schematic of an infrared thermography device suitable for use in imaging thermogenesis in a living animal. 1=Infrared camera; 2=isothermal chamber; 3=heating pad ( $37^\circ\text{C}$ ); 4=computer interface; 5=intrascapular brown adipose tissue (IBAT).

Figures 3A and 3B. Infrared thermography data of yeast (Fig. 3A; 384-well plate) and human adipocyte cultures (Fig. 3B; 96-well plate) treated with rotenone and FCCP presented using a gray scale index or using graphical numerical axes (Rotenone =  $\bullet$ ; FCCP =  $\blacktriangle$ ).

Figures 4A-4C. Presentation of thermographic analysis of yeast cells expressing uncoupling protein 2 (UCP2) (Figs. 4A and 4B) and molecular analysis of UCP2 expression in the yeast cells (Fig. 4C).

Figure 5. Infrared thermographic analysis of thermogenesis in chinese hamster ovary cells (CHO) overexpressing the  $\beta_3$ -AR receptor in the presence of forskolin ( $\blacksquare$ ) or isoproterenol ( $\bullet$ ) (blank =  $\blacktriangle$ ).

Figure 6. Analysis of triglyceride accumulation ( $\bullet$ ) and heat production ( $\blacktriangle$ ) in adipocytes.

Figure 7. Infrared thermographic image of differentiating adipocytes representing a dose response curve for several peroxisome

proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) agonists in the presence of insulin and 9-*cis* retinoic acid.

Figures 8A and 8B. Presentation summarizing the infrared thermographic analysis of the intrascapular area of *ob/ob* mice treated with a PPAR $\gamma$  agonist, GW1929 (Fig. 8A), and the correlation between thermographic signal and the minimal effective dose (MED) of various PPAR $\gamma$  agonists in mice (Fig. 8B) ( $R^2=0.954$ ).

Figure 9. Presentation showing rotenone sensitivity of  $\beta$ -AR agonist (50nM)-induced thermogenesis in adipocytes.

Figure 10. Presentation of infrared thermographic analysis at 10 minutes of primary human epithelial cells (HUVEC cells) in the presence (solid gray bar) and absence (hatched bar) of vascular endothelial growth factor (VEGF).

Figure 11. Infrared thermographic analysis of a chemical reaction following NaOH addition to HCl.

Figures 12A and 12B. Infrared thermographic analysis of catalytic reactions on combi-chem beads in a 25 ml beaker (Fig. 12A) (1=bulk solvent); 2=active beads; 3=O-ring) or in a 96 well microtiter plate (Fig. 12B; inactive and active beads).

Figures 13A-13D. Infrared thermographic analysis of a metered dose inhaler (MDI) device (Fig. 13A; actuations 0, 1 and 5) during and after 5 consecutive actuations (Fig. 13B). Presentation of infrared thermographic analysis of nude mice treated with the inhalant, Albuterol

(Fig. 13 C), and subsequent quantitation of the thoracic areas showing the kinetics of Albuterol activity (Fig 13D).

Figures 14A and 14B. Presentation of infrared thermography analysis of intrascapular thermogenesis of mice showing a dose-response curve and kinetic data after treatment with a  $\beta_3$ -AR agonist (1.0 mg/kg =  
5 -●-; 0.1 mg/kg = -■-; 0.01 mg/kg = -▲-).

Figures 15A and 15B. Infrared thermographic analysis of intrascapular thermogenesis of *ob/ob* mice -- dose-response (Fig. 15A) and kinetic data (Fig. 15B) after treatment with monoamine reuptake  
10 inhibitor GW473559A (10.0 mg/kg = -●-; 5.0 mg/kg = -■-; 1.0 mg/kg = -▲-).

Figure 16. Graph correlating intrascapular thermogenesis and weight reduction in mice treated with a  $\beta_3$ -AR agonist (IBAT thermogenesis = bar, 2-week weight reduction = -●-) ( $R^2=0.97$ ).

15 Figure 17. Graph demonstrating the use of infrared thermography to characterize the influence of genetic background and diet on  $\beta_3$ -mediated thermogenesis of 19 week old rodent animal models (low fat diet = solid gray bar; high fat diet = hatched bar (animals on diet 14 weeks)) on intrascapular thermogenesis.

20 Figures 18A and 18B. Infrared thermographic analysis of diet-induced thermogenesis in humans. Fig. 18A shows dosal temperature versus time; Fig. 18B shows mean differences in dosal temperature of 2 males and 1 female (mean=3-5 different days/subject) before and after lunch.

Figure 19. Presentation of infrared thermographic analysis of drug-induced thermogenesis in humans (subject A and subject B) treated with ephedrine (left=0 minutes; right =60 minutes) (dose = 0.6-0.7 mg/kg -  $\Delta T^{\circ}\text{C}$  (whole back = 0.63 for subject A and 0.46 for subject B)).

- 5        Figure 20. Infrared thermographic characterization of interactions between two drugs, the  $\beta_3$ -AR agonist CGP12177 (1 mg/kg - I.P.) and the PPAR $\gamma$  agonist GW1929, that influence intrascapular thermogenesis in *ob/ob* mice (1=intrascapular region).

- 10       Figures 21. Infrared thermographic analysis of VEGF peptide-induced thermogenic activity in nude mice (1=VEGF; 2=control).

Figure 22. Infrared thermographic analysis of the effect of anti-VEGF antibody on tumor temperature.

- 15       Figures 23A and 23B. Infrared thermographic analysis of the effect of etoposide treatment (6 mg/kg) in utero on hair loss in newborn mouse pups. Fig. 23A shows the thermographic image obtained at day 0, day 3 and day 5. Fig. 23B shows torso  $\Delta T(^{\circ}\text{C})$  versus time post dosing (days) (back = open bar, front = solid gray bar).

Figure 24. Infrared thermographic analysis of Pinacidil-induced changes in thermogenesis in rat genitalia (2 hours post PO dosing).

- 20       Figures 25A and 25B. Infrared thermographic analysis of limb inflammation induced by proteoglycan polysaccharide (PGPS) (Fig. 25A; 1=non-arthritic limb; 2=arthritic limb) and reduced by 6 mg/kg p.o. prednisolone (Fig. 25B) (Vehicle = HPMC +0.1% TW80; p.o.).

### DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a method of using infrared thermography as a tool to monitor temperature changes that occur during molecular interactions, including those that occur in isolated cells, tissues, plants, animals (e.g., man). Infrared thermography can be used to analyze the effects of various agents on heat production in a variety of cell, tissue, plant, and animal types, during enzyme catalysis, and, more generally, during ligand interaction with a binding partner. Thus, infrared thermography can be used to identify agents suitable for treating various diseases, disorders, and conditions, including those involving altered thermogenic responses.

Generally, the designation "infrared radiation" refers to electromagnetic radiation having a wavelength of between about 2.5 and about 50 microns or, expressed differently, that having a frequency of between about 200 and about 4000 inverse centimeters ( $\text{cm}^{-1}$  or "wave numbers"). As understood by those familiar with infrared (IR) radiation and the IR spectrum, the frequencies of electromagnetic radiation generally characterized as infrared are emitted or absorbed by vibrating molecules, and such vibrations generally correspond to the thermal state of a material in relation to its surroundings. All solid bodies whose temperatures are above absolute zero radiate some infrared energy, and for temperatures up to about 3500°K (3227° Celsius, 5840° Fahrenheit), such thermal radiation falls predominantly within the infrared portion of the electromagnetic spectrum. There thus exists a rather straightforward relationship between the temperature of a body and the infrared radiation which it emits. In the present invention, the monitoring of radiation in the



range of 3-100 microns is preferred, 3-15 microns being more preferred and 3-12 microns being most preferred (e.g., 6-12 microns).

As further understood by those familiar with electromagnetic radiation, wavelengths below  $2.5\text{ cm}^{-1}$  are considered as the "near IR" portion of the electromagnetic spectrum, and represent vibrational "overtones" and low level electronic transitions. The designation "infrared" as used herein is not intended to encompass the "near IR" portion of the electromagnetic spectrum.

In accordance with the invention, an infrared imaging system, advantageously, a high resolution infrared imaging system, is used to monitor real time heat output, for example, from cells or tissues in culture or from laboratory animals, with images provided by a central processing unit for data analysis (see Figs. 1 and Fig. 2 and Examples that follow). An example of a suitable system is that produced by FLIR Infrared Systems (the AGEMA 900 or QWIP SC3000). Similarly, the temperature can be measured using a non-contact infrared thermometer such as that produced by Linear Laboratories (the C-1600MP). Neither the AGEMA 900 nor the C-1600MP has heretofore typically been used in to practice a method of the present invention. However, these apparatuses can be adapted by techniques known to those skilled in the art to measure changes in temperature in the range of, for example,  $5.0^{\circ}\text{C}$ , or higher, to  $0.000001^{\circ}\text{C}$  or lower, preferably,  $1.0^{\circ}\text{C}$  to  $0.00001^{\circ}\text{C}$  or  $0.5^{\circ}\text{C}$  to  $0.0001^{\circ}\text{C}$ , more preferably,  $0.3^{\circ}\text{C}$  to  $0.0005^{\circ}\text{C}$  or  $0.25^{\circ}\text{C}$  to  $0.001^{\circ}\text{C}$ , most preferably,  $0.2^{\circ}\text{C}$  to  $0.002^{\circ}\text{C}$  (see Fig. 1 and Fig. 2 and Examples that follow).

In one embodiment, the present invention relates to a method of monitoring the effect of an agent on thermogenesis in isolated cells,

tissues, or in animals (including primates (e.g., humans)). The method comprises: i) measuring the heat produced by the cells, tissues, or a given surface area of animals before exposure to the agent using infrared thermography, ii) exposing the cells, tissues, or animal to the agent (e.g.,  
5 by adding the agent to culture medium in which the cells or tissues are maintained/grown or by treating the animal with the agent using standard delivery techniques), iii) measuring the heat produced by the cells, tissues, or animals during and/or after treatment with the agent using infrared thermography, and iv) comparing the measurements obtained in  
10 steps (i) and (iii), wherein an agent that results in a lowering of the temperature of the cells, tissues or animals is an inhibitor of thermogenesis and an agent that results in an elevation of the temperature is a stimulator of thermogenesis.

Cells that can be monitored in accordance with the invention  
15 include isolated naturally occurring cells (including primary cultures and established cell lines) and engineered cells (e.g., isolated engineered cells). The cells can be in suspension or attached to a solid support either as a monolayer or in multilayers. Examples of suitable supports include plastic or glass plates, dishes or slides, membranes and filters, flasks,  
20 tubes, beads and other related receptacles. Advantageously, plastic multiwell plates are used, 96-well and 384-well microtiter plates being preferred. While preferred cell titers range between 100 to 100,000 cells/cm<sup>2</sup> for adherent cells and 100 to 1,000 cells/ $\mu$ l in the case of suspension cultures, potentially any cell number/concentration can be  
25 used.

Isolated naturally occurring cells that can be monitored in accordance with the present method include eucaryotic cells, preferably

mammalian cells. Primary cultures and established cell lines and hybridomas (such as those available from the American Type Culture Collection) can be used. Specific examples include cells or tissues derived from fat (e.g., adipocytes and precursors thereof), muscle (e.g., myotubes, myoblasts, myocytes), liver (e.g., hepatocytes, Kupffer cells), the digestive system (e.g., intestinal epithelial, salivary glands), pancreas (e.g.,  $\alpha$  and  $\beta$ -cells), bone marrow (e.g., osteoblasts, osteoclasts, and precursors thereof), blood (e.g., lymphocytes, fibroblasts, reticulocytes, hematopoietic progenitors), skin (e.g., keratinocytes, melanocytes), amniotic fluid or placenta (e.g., chorionic villi), tumors (e.g., carcinomas, sarcomas, lymphomas, leukemias), brain (e.g., neurons, hypothalamus, adrenal and pituitary gland), the respiratory system (e.g., lung, trachea), connective tissue (e.g., chondrocytes), eye, kidney, heart, bladder, spleen, thymus, gonads, thyroid and other organs involved in endocrine regulation. There are no restrictions on the cell types that can be used. The present method is applicable to cells derived from plants, fungi, protozoans, and the monera kingdom (e.g., bacteria). The cells can be cultured using established culture techniques and culture conditions can be optimized to ensure viability, growth and/or differentiation, as appropriate.

Engineered cells that can be monitored in accordance with the present method include cells engineered to produce or overproduce proteins involved directly or indirectly in temperature regulation, energy balance and fuel utilization, growth and differentiation and other aspects of physiology or metabolism that alter heat generated by cells. Such cells can be engineered prokaryotic cells (kingdom monera: e.g., *E. coli*),

engineered higher or lower eucaryotic cells, or cells present in or isolated from transgenic animals. Examples of higher eucaryotic cells (*e.g.*, from the plant and animal kingdoms) include cell-lines available from the American Type Culture Collection (*e.g.*, CV-1, COS-2, C3H10T1/2, HeLa, and SF9). Examples of lower eucaryotic cells include fungi (*e.g.*, yeast) and protozoans (*e.g.*, slime molds and ciliates). The cells or transgenic animals can be engineered to express any of a variety of proteins, including but not limited to nuclear receptors and transcription factors (*e.g.*, retinoid receptors, PPARs, CCAAT-Enhancer-Binding Proteins (CEBPs), polymerases), cell surface receptors (*e.g.*, transmembrane and non-transmembrane receptors, G protein-coupled receptors, kinase-coupled receptors), membrane transporters and channels (*e.g.*, uncoupling proteins, sugar transporters, ion channels), signal transduction proteins, (*e.g.*, phosphodiesterases, cyclases, kinases, phosphatases), and viruses (*e.g.*, AIDS, herpes, hepatitis, adeno). Engineered cells can be produced by introducing a construct comprising a sequence encoding the protein to be expressed and an operably linked promoter into a selected host. Appropriate vectors and promoters can be selected based on the desired host and introduction of the construct into the host can be effected using any of a variety of standard transfection/transformation protocols (see Molecular Biology, A Laboratory Manual, second edition, J. Sambrook, E.F. Fritsch and T. Maniatis, Cold Spring Harbor Press, 1989). Cells thus produced can be cultured using established culture techniques and culture conditions can be optimized to ensure expression of the introduced protein-coding sequence.

The present method can be used to identify, characterize, rank, and select agents (*e.g.*, drugs or drug candidates) suitable for use in

treating various diseases, disorders or conditions based on potency, selectivity, efficacy, pharmacokinetics and pharmacodynamics of the agent in various cell-free, cell, tissue, plant, animal, and human-based thermogenesis assays. For example, a test agent can be screened using

5 infrared thermography for its potential as a catabolic or anabolic drug. Cultured cells (e.g., primary cells, such as adipocytes or yeast, or cell-lines, such as C3H10T1/2 mesenchymal stem cells, osteoblasts, or adipocytes), plants, animals, or humans (including patients in clinical studies during pharmaceutical development) can be treated with the test

10 agent followed by infrared thermography to measure changes in heat signature. Agents that enhance thermogenesis (cellular heat production) are potentially useful as catabolic drugs and agents that suppress thermogenesis are potentially useful as anabolic drugs.

In addition to changes in metabolism, alterations in thermogenesis

15 can reflect changes in growth and differentiation. Thus, the present method can be used to identify, characterize, rank, and select agents (e.g., drugs or drug candidates) suitable for use in treating or preventing diseases, disorder or conditions associated with changes in metabolism, toxicity, cellular growth, organ development, and/or differentiation.

20 Examples of pathophysiologies potentially amenable to treatment with anabolic agents identified with infrared thermography include anorexia, alopecia, auto-immunity, cachexia, cancer, catabolism associated with aging, diabetes, graft rejection, growth retardation, osteoporosis, pyrexia, bacterial and viral infections. Examples of

25 diseases, disorders or conditions potentially amenable to treatment with catabolic agents identified with infrared thermography include diseases, disorders or conditions associated with obesity (e.g., hypertension,

dyslipidemias, and cardiovascular diseases) and diseases, disorders or conditions associated with accelerated growth (e.g., cancer, gigantism, certain viral infections). The pathophysiologies amenable to treatment using agents identified with infrared thermography are not limited to those commonly associated with changes in anabolism or catabolism (e.g., metabolic diseases). The approach is also applicable to other diseases, disorders and conditions including male erectile dysfunction (MED), inflammation, hypertension, gastrointestinal diseases, behavioral disorders (CNS diseases), and diseases associated with changes in blood flow. There are no restrictions on the pathophysiologies that can be analyzed in accordance with the present invention in pharmaceutical research and development (e.g., analysis of drug potency, efficacy, toxicity, pharmacokinetics and pharmacodynamics).

In accordance with the present invention, the binding of a ligand (proteinaceous or nonproteinaceous (e.g., a nucleic acid)) to a binding partner (proteinaceous or nonproteinaceous (e.g., a nucleic acid)), where binding elicits a thermogenic response, can be monitored using infrared thermography. The ligand and/or binding partner can be in a cell or in a cell-free environment (e.g., a solution). The ligand and/or binding-partner can be a synthesized chemical entity that does not normally exist in nature, or the ligand and/or binding-partner can be a naturally occurring entity such as a naturally occurring protein, nucleic acid, polysaccharide, lipid, hormone, or other naturally occurring substance or cell. The effect of a test agent (e.g., potential ligand) on heat generated by its binding partner can be measured using infrared thermography. One suitable method comprises: i) measuring the heat produced by the binding-partner, ii) adding test agent to the binding-partner, iii) measuring heat

produced after mixing the potential ligand (test agent) and binding-partner, and iv) comparing the measurements in (i) and (iii), wherein an agent that alters heat generation is a ligand for the binding partner. Additionally, test agents can be screened for their ability to alter the thermogenic response  
5 resulting from the binding of the ligand to its binding-partner. Such agents can be allosteric regulators, agonists, or antagonists of the ligand and/or binding partner. Such a screen can comprise: i) measuring the heat produced upon addition of the first member of the binding pair (ligand or binding-partner) to the second member of the binding-pair using infrared  
10 thermography, and ii) measuring the heat produced upon addition of the first member of the binding pair, the second member of the binding-pair and test agent, and iii) comparing the measurement in (i) with that in (ii), wherein an agent that alters the heat generation observed upon addition of the ligand to its binding partner is a modulator of that interaction, for  
15 example, by binding to either or both members of the binding pair.

Further in accordance with the present invention, agents can be screened for their ability to modulate the rate of catalysis of a particular enzyme. The method can comprise measuring the heat produced upon addition of an enzyme to its substrate using infrared thermography and  
20 measuring the heat produced upon addition of a test agent, the enzyme, and its substrate, and comparing the results. An agent that alters heat production can be an enzyme inhibitor or activator. Controls that can be run in accordance with such a method include measuring the heat produced upon addition of the enzyme to the test compound (in the absence of substrate)  
25 and upon addition of the substrate to the test compound (in the absence of the enzyme). Such controls permit determination of the effects on heat

production from the respective additions. Using such an approach, test agents can be screened for their ability to behave as substrates. Such agents can increase heat production when mixed with enzyme in the absence of any other known substrate.

5 In another embodiment, the present invention relates to agents identified using the above-described assays. The agents identified in accordance with the above assays can be formulated as pharmaceutical compositions. Such compositions comprise the agent and a pharmaceutically acceptable diluent or carrier. The agent can be present in  
10 dosage unit form (e.g., as a tablet or capsule) or as a solution, preferably sterile, particularly when it is to be administered by injection. The dose and dosage regimen will vary, for example, with the patient, the agent and the desired effect. Optimum doses and regimens can be determined readily by one skilled in the art.

15 In another embodiment, the present invention relates to a method of monitoring the effects of environmental changes (e.g., diet) and/or genetic background on thermogenesis in various organisms (animals, plants, tissues, and cells). The method can comprise: i) measuring heat produced either by an organism, using infrared thermography, under  
20 different environmental conditions (e.g., fed different diets: high or low fat, protein, or carbohydrate diets) or by organisms with different genetic backgrounds (e.g., inbred animals, populations), ii) exposing the organism(s) to various agents (e.g., placebos or thermogenic agents; including untreated controls), iii) measuring the heat produced by the  
25 organism(s) after treatment with the agent using infrared thermography, iv) comparing the measurements in steps (i) and (iii), to determine the influence of environmental changes and genetic background.



The present method can be used to identify, predict, characterize, rank, and select how different environments (e.g. diet) or genotypes can influence basal or agent-induced thermogenesis. There are no restrictions as to the population, species, strain, sex, or age of the organisms or the diet or environmental conditions that can be used. The organisms can be naturally occurring (e.g., house mouse), inbred (e.g., AKR/J mice), or engineered (e.g., transgenic mice). The method can comprise measuring the heat produced using infrared thermography upon changing the diet, circadian cycle, diurnal rhythm, altitude, barometric pressure, humidity, temperature, noise, sleep status, physical or mental stress and injury of the cell or organism. Diets can be poorly defined (e.g., cafeteria diets) or well characterized (e.g., laboratory chow). The organisms can be fed on scheduled diets or *ad libitum*. The agents that alter thermogenesis can be naturally occurring or synthetic, known or unknown, safe or toxic, and anabolic, catabolic, or without effect.

Environmental (dietary or otherwise) changes, genotypes, or agents that enhance thermogenesis (body heat production) are potentially useful for identifying catabolic states. Environmental changes, genotypes, or agents that suppress thermogenesis (body heat production) are potentially useful for identifying anabolic states.

In another embodiment, the present invention relates to a method of monitoring drug-drug interactions in various organisms (humans, animals, plants, tissues, and cells). The method comprises: i) measuring the heat produced by the organism (cells, etc.), using infrared thermography, before exposure to the agent(s), ii) exposing the organism (cells, etc.) to a single agent and to multiple agents (e.g., by adding to culture medium or dosing by injection, gavage, topical application, etc.), iii) measuring the heat produced by the organism (cells, etc.) after

treatment with a single agent and after treatment with multiple agents, using infrared thermography, iv) determining the differences in heat produced in steps (i) and (iii) and comparing the differences in heat produced after exposure to single agents with the heat produced after exposure to combined agents. A difference in the heat produced after exposure to multiple agents (as opposed to single agents) indicates that the agents interact or are eliciting a thermogenic response.

As indicated above, agents that result in a change in thermogenesis when used in combination, relative to when used singly, are proposed to be involved in pharmacodynamic drug-drug interactions. Such interactions can be potentially toxic or beneficial to the organism, tissue, or cells. As such, infrared thermography can be used to identify, predict, characterize, rank, and/or select how different agents (e.g., drugs) interact with each other. There are no restrictions to the type and number of agents, cells, tissues, and organisms that can be used. The agents can be naturally occurring, synthetic, agonists, antagonists, inhibitors, activators, safe, toxic, anabolic, catabolic, known, or unknown. The cells, tissues, and organism can be derived from plants, animals (e.g., man), fungi, protozoans, or monera. Infrared thermography can be used to measure the heat produced by cells, tissues, and/or organisms upon changing various pharmacokinetic and pharmacodynamic parameters, including altering the duration of exposure, the concentration of agent(s), pharmaceutical compositions, and number of agents used.

In a further embodiment, the present invention relates to a method of monitoring hair loss (alopecia) and regrowth. As such, infrared thermography can be used to identify, predict, characterize, rank, and/or select how different treatments or environmental stimuli alter hair growth.

There is no restriction on the types of treatment or stimuli that may alter hair loss and growth. The types of treatments can include diet, exercise, pharmacological, radioactive, or surgical intervention and can be invasive or noninvasive. The stimuli for altering hair growth can be present

5 naturally in the environment (e.g., radon gas) or a result of environmental contamination (pollution, such as pesticides). Thus, infrared thermography can be used to monitor the safety, potency, and efficacy of various treatments (natural or artificial) on hair loss and growth.

In another embodiment, the present invention relates to a method

10 of evaluating safety profiles of pharmacologic agents. In accordance with this embodiment, various proteins (e.g., cytochrome P450s etc.), organelles (e.g., microsomes, etc.), cells, tissues, and organ types targeted by an agent can be isolated, treated with varying concentrations of the agent and heat production monitored using infrared thermography.

15 This method can comprise: i) determining the potency and efficacy of an agent on stimulating or inhibiting heat production in the desired target (e.g., a protein, organelle, cell, tissue, or organ involved in the therapeutic effect of an agent), ii) determining the potency and efficacy of an agent on stimulating or inhibiting heat production in an undesirable target (e.g., a

20 protein, organelle, cell, tissue, or organ involved in a toxic effect of an agent), iii) determining the selectivity of the agent by comparing the potency and efficacy in steps (i) and (ii). Pharmacological agents that show increased selectivity between the various targets (e.g., protein, organelle, cell, tissue, and/or organ), can be expected to have improved

25 safety profiles. Consistent with this embodiment, the effects of varying the concentration of the test agent on heat generated by binding-partners and/or enzyme catalysis can be used to evaluate the selectivity and safety

profile against multiple targets. Optimum selectivity between desirable and undesirable targets (e.g., cell types, binding-partners, or enzymes) can be determined readily by one skilled in the art.

In another embodiment, the present invention relates to a method  
5 of evaluating the physical state and/or amount of a compound(s). In accordance with this embodiment, the physical state of a compound can be determined using this method as it relates to a compound changing its physical properties of going from a solid (i.e. frozen liquid) to a liquid (i.e. melting), a liquid into a solid (i.e. crystallization), a liquid into a gas (i.e.  
10 evaporation, vaporization), a solid into a gas (i.e. sublimation). This embodiment can be applied but is not limited to compounds in open vessels, closed systems, pressurized vessels (i.e. inhalants). The amount of a liquid can be measured using the present invention. Consistent with this embodiment, each varying amount of the test agent generates a  
15 unique heat profile whereby the amount of agent present can be measured by its unique heat characteristics.

It will be appreciated from a reading of the foregoing that the invention has applicability in connection with virtually any animal or animal tissue. For example, the above-described methods can be applied to  
20 mammals, including primates (e.g. humans) and any of the commonly used laboratory animals (e.g., rats, mice, hamsters, guinea pigs and rabbits) as well as to birds, amphibians and reptiles and insects.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

### EXAMPLES

The following experimental protocols and details are referenced, whole or in part, in the non-limiting Examples that follow.

#### Adipocytes

- 5 Human subcutaneous adipocytes were purchased from Zen-Bio, Inc. (Research Triangle Park, NC). C3H10T1/2 clone 8 fibroblasts were differentiated into adipocytes as previously described (Lenhard et al, Biochem. Pharmacol. 54:801-808 (1997), Paulik and Lenhard, Cell Tissue Res. 290:79-87 (1997)). After 7 days in culture, triglyceride accumulation  
10 was determined by adding lipoprotein lipase and GPO-Trinder reagent (assay kit 337-B, Sigma Diagnostics, St. Louis, MO) to the cells (50  $\mu$ l/cm<sup>2</sup>) and incubating the lysates at 37°C for 2 hours. The optical density was measured using a spectrophotometer set at a wavelength of 540 nm. Lipolysis was measured as previously described (Lenhard et al, Biochem.  
15 Pharmacol. 54:801-808 (1997)).

#### Cloning of UCP2 and yeast transformation

- Human skeletal muscle cDNA (#7175-1) was purchased from Clontech (Palo Alto, CA). UCP2 specific-sequences were PCR amplified  
20 from the sample using oligonucleotide primers that matched the 5' and 3' ends of a published sequence (GenBank U82819). Vent polymerase was used (New England Biolabs, Beverly, MA) in a standard reaction mixture with 2 mM MgSO<sub>4</sub> and 5% DMSO. The cycle parameters were 94°C for 1 min., 55°C for 1 min., and 72°C for 1 min., repeated 29 times. The sample  
25 was passed over an S-400 spin column (Pharmacia, Piscataway, NJ) prior

to ligation in a vector for transformation of *E. coli*. The authenticity of the clone was confirmed by DNA sequence analysis. For the experiments in yeast, the UCP2 coding sequence was amplified by PCR using primers with the sequence AAAAAACCCCGGATCGAATTCATGGTTGGGTTC

5 AAGGCCA (SEQ ID NO:1) (sense) and  
CATTGTTCTTATTCAGTTACTCGAGTTAGAAGGGAGCCTCTCGGGA  
(SEQ ID NO:2) (antisense) followed by a second PCR using primers with  
the sequence TTAACGTCAAGGAGAAAAACCCCGGATCG (SEQ ID  
NO:3) (sense) and GAAAGGAAAAACGTTTCATTGTTCTTATTCAG  
10 (SEQ ID NO:4) (antisense). The PCR product was cloned into pYX233  
(R&D Systems) by homologous recombination in yeast strain W303 (a/a  
homozygous for *ade2-1*, *his3-1,15* *leu2-3,112*, *trp1-1*, *ura3-1*). Yeast  
transformants were selected on BSM-trp agar (Bio 101, Vista, CA). The  
correct UCP2 sequence was verified by sequencing plasmids back-  
15 extracted from yeast to *E. coli*.

For analysis of UCP2 expression and thermogenesis, yeast  
containing the expression plasmids were propagated for 24 hours in BSM-  
trp broth, washed once and inoculated at  $A_{600}=0.001$  into YP containing 2%  
DL-lactic acid, pH 4.5, 1% ethanol, 0.1% casamino acids and 40 mg/ml  
20 adenine. Cultures were induced 16 hours later by adding galactose to  
0.4% (w/v). To assess UCP2 expression, yeast ( $A_{600} = 0.1$ ) were lysed in  
NuPAGE sample buffer (Novex, San Diego, CA) containing 5%  $\beta$ -  
mercaptoethanol and soluble proteins separated on 10% NuPAGE gels  
(Novex, San Diego, CA). A synthetic peptide using the UCP2 amino acid  
25 sequence LKRALMAAYQSREAPF (SEQ ID NO:5) was synthesized and  
used to generate antibodies [Zeneca, Wilmington, DE]. Western blot

analysis was performed following published methods (Paulik and Lenhard, Cell Tissue Res. 290:79-87 (1997)).

#### Experimental animal protocol

5           Age and weight matched male *+ob/+ob* mice (Jackson Labs, Bar Harbor, ME) were housed 5 animals/cage at 72° F and 50% relative humidity with a 12 hr light and dark cycle, and fed chow diet (NIH R&M/Auto 6F-Ovals 5K67, PMI Feeds® Inc., Richmond, Indiana).

10           Animals starting at 41 days of age were orally gavaged once daily (8:00-9:00 AM) with 0.05M N-methylglucamine (control) and 5 mg/kg GW1929 in 0.05M N-methylglucamine. After 2 weeks of dosing, the animals were dosed (intraperitoneal) with 1 mg/kg of CGP12177A, and the animals were anesthetized with isoflurane. Infrared thermographic images and temperature calculations were recorded using an Agema Thermovision  
15           900 Infrared System. The data was calculated as the mean and standard error from experiments performed on  $\geq 6$  animals per treatment group.

This research complied with the principles of laboratory animal care (NIH publication No. 86-23, revised 1985) and company policy on the care and use of animals and related codes of practice.

20           Male AKR/J, C57BL/6J, and SWR/J mice were purchased from 4-8 weeks of age from Jackson Laboratories (Bar Harbor, ME). Mice were fed high and low fats diets containing high sucrose as defined by Surwit et al., (Metabolism 44(5):645-651 (1995)). After 14 weeks on these diets, the intrascapular fur was shaved, the animals were dosed (intraperitoneal) with  
25           1 mg/kg of BRL37344, and the animals were anesthetized with isoflurane. Nude mice (BALB/C) were 6-8 weeks old and were dosed

with inhalant two times before thermal profiling. The Lewis rat strain (200-250g) were used for both the MED and inflammation experiments. Infrared thermographic images and temperature calculations were recorded using an Agema Thermovision 900 Infrared System.

5

#### Infrared thermography

Heat generation was measured using a Stirling cooled Agema Thermovision 900 Infrared System AB (Marietta, GA) equipped with a SW  
10 Scanner and 40° x 25° lens which detects a 2-5.4 micron spectral response. The scanner had an internal calibration system with an accuracy of 0.08°C. The focal distance was 6 cm. Images were captured using a recurs function set at 16 or an averaging function set at 32. The data was analyzed using OS-9 advanced systems and ERIKA 2.00  
15 software according to the manufacturer's specifications (FLIR Infrared Systems AB, Danderyd, Sweden). Thermography of adipocytes was performed by maintaining the ambient temperature of the cultured cells at  $37 \pm 0.02^\circ\text{C}$  using a Queue Systems Inc. (Parkersburg, W.V.) incubator, model QWJ500SABA. Spectral analysis of yeast was performed at  $30 \pm$   
20  $0.02^\circ\text{C}$  using the same incubator system. After treating the cells with experimental agents (e.g., rotenone etc.), the temperature was equilibrated for 10 minutes in the incubator before measuring real time thermogenesis for all microtiter plate applications. Various color scales in the visible wavelength were used to depict the temperature fluctuations of  
25 the recorded images. Although temperature scales are constant, the color scale images are variable and can be adjusted with level and span controls.



Various infrared detection system parameters were optimized using both yeast and cultured adipocytes. Cell titer experiments using yeast revealed a lower detection limit at a density of  $A_{600} = 0.01$  and a maximal thermogenic response at a density of  $A_{600} = 0.1-0.2$ . Whereas thermal activity of C3H10T1/2 cell suspensions was detected as low as  $8 \times 10^3$  cells/well, the greatest signal was obtained at  $1 \times 10^5$  cells/well. As expected, the maximal thermogenic response of adherent adipocytes was observed using confluent cells. A comparison of microtiter plates revealed 384-well formats were best for measuring thermogenesis of yeast suspensions while 96-well formats were most suitable for culturing adherent adipocytes. The outer wells of the culture plates were omitted from the detection system because increased thermal conductance occurred at the edge of the plates. Larger diameter wells (*i.e.*,  $>1$  cm) were less satisfactory because a meniscus effect was observed that resulted in uneven thermal conductance. Further, the amount of media per well was critical, since too much media decreased the signal and too little media created a meniscus resulting in increased background noise. The best results were obtained using 50  $\mu$ l/well using both 96-well plates containing adherent adipocytes and 384-well plates containing yeast suspensions. Enclosure of the detection system and objects that were being profiled was essential for minimizing changes in temperature and reflectivity (*i.e.*, thermal noise) which result from air currents and light, respectively. Materials that exhibited low reflective properties (*e.g.*, anodized aluminum), especially those materials that were in view of the infrared detector, were ideal for minimizing extraneous thermal noise during thermal profiling (*e.g.*, chamber plates, etc.). Finally, increasing the

temperature equilibration time (*i.e.*, > 10 min) improved the signal to noise ratio.

### EXAMPLE 1

#### 5                    Design of an apparatus for infrared thermography

10                    The methods of the present invention utilize an apparatus that, advantageously, consists of a high resolution infrared imaging system and a central processing unit with appropriate software for data analysis. An example of a suitable system is that produced by FLIR Infrared Systems (the AGEMA 900) or the non-contact infrared thermometer (C-1600MP) produced by Linear Laboratories. Figure 1 shows a schematic diagram of such an apparatus for infrared thermography of cell-free systems or cell culture. The isothermal chamber, constructed from a non-reflective material that provides a heat sink to dampen out temperature fluctuations (e.g., anodized aluminum) minimizes thermal noise (e.g., reflection and air currents) from the culture plates and surrounding environment. A plate holder can be placed within the isothermal chamber to maintain thermal uniformity across the plate. Use of an incubator also prevents fluctuations in the surrounding temperatures and improves cellular responses and viability. In the schematic shown in Figure 1, the camera monitors real time heat production from the cells in culture with images recorded by a central processing unit for data capture and software analysis tools for further data analysis. Figure 2 shows a schematic diagram of an apparatus designed for infrared thermography of the intrascapular region of mice. This apparatus shares features with the apparatus in Figure 1,

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including the infrared camera, isothermal chamber and computer interface. It can also include an infrared screening platform with anesthetic manifolds for maintaining the animals under anesthesia, a tightly regulated heating block, for example, made out of anodized  
5 aluminum, and an isothermal chamber to maintain air currents at a minimum.

### EXAMPLE 2

#### Infrared Thermography in Cultured Cell Systems (*e.g.*, adipocytes and yeast): Effect of Small Molecules on Thermogenic Activity

10 As part of the initial effort to validate the use of infrared thermography to monitor mitochondrial heat production, human adipocytes cultured in 96-well microtiter plates and yeast suspended in 384-well microtiter plates were treated with rotenone or carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP). Rotenone is an inhibitor of  
15 mitochondrial electron transport (Ahmad-Junan et al, Biochem. Soc. Trans. 22:237-241 (1994)) while FCCP is an uncoupler of mitochondrial respiration (Terada, Biochem. Biophys. Acta 639:225-242 (1981)). Subsequently, the heat produced from the cells was measured using an Agema Thermovision 900 Infrared Imaging System (Fig. 1). As shown in  
20 the dose response assays in Fig. 3, rotenone treatment inhibited thermogenesis in yeast (Fig. 3A) and human adipocytes (Fig. 3B). In contrast, FCCP stimulated heat production in both cell types relative to untreated cells. Kinetic analysis revealed that changes in heat production were detectable 10 minutes after treating the cells with either agent.

These results validate the use of infrared thermography for measuring the dose-dependent thermogenic effects of small molecules (e.g. FCCP, rotenone) on cells in culture or in cell-free systems.

### EXAMPLE 3

#### 5                   Effect of Protein Overexpression on Thermogenesis

In rodents, intrascapular brown adipose tissue (IBAT) is an important site for adaptive thermogenesis (Himms-Hagen, Proc. Soc. Exp. Biol. Med. 208:159-169 (1995)) and is located in the intrascapular region of rodents. This tissue contains abundant mitochondria which express the  
10   anion transporter, uncoupling protein (UCP1, formerly known as UCP; Ricquier et al, FASEB J. 5:2237-2242 (1991)). UCP1 uncouples oxidative phosphorylation from respiration in IBAT resulting in generation of heat instead of ATP. Although UCP1 is not abundant in Homo sapiens, UCP2 (Fleury et al, Nat. Genet. 15:269-272 (1997)) is abundantly expressed in  
15   humans. UCP2 mRNA is ubiquitously expressed and its expression is altered in obesity (Enerback et al, Nature 387:90-94 (1997)). Further, the antidiabetic thiazolidinediones (e.g., troglitazone) increase UCP2 expression, possibly by activating the nuclear receptor PPAR $\gamma$ , suggesting UCP2 plays a critical role in regulating energy balance  
20   (Shimabukuro et al, Biochem. Biophys. Res. Commun. 237:359-361 (1997)).

Although UCP1 regulates mitochondrial-mediated thermogenesis in rodents, there is no direct evidence that UCP2 plays a similar role. In order to evaluate UCP2's role in thermogenesis and validate the use of  
25   infrared thermography to detect physiological changes in cells engineered

to express foreign proteins, the UCP2 gene was cloned from a human cDNA library and expressed in yeast using a galactose-inducible expression system. As shown in Fig. 4A, expression of UCP2 in yeast resulted in increased thermogenesis relative to cells lacking UCP2. As expected, treatment of the cells with rotenone inhibited UCP2-mediated thermogenesis (Figs. 4A and 4B). For reference, it was confirmed that UCP2 was expressed in these cells by Western blot analysis (Fig. 4C). Peak expression and thermogenesis was observed 3-4 hours after inducing UCP2 synthesis with galactose. The results validate the use of infrared thermography as a tool to measure the effects of various molecules (e.g., reporter genes such as UCP2) on mitochondrial-mediated thermogenesis in cell models (e.g. fungi (e.g., yeast), SF9, CHO, Neurospora, etc) engineered to express foreign proteins.

In order to further validate the use of infrared thermography to detect physiological changes in cells engineered to overexpress foreign proteins, the beta 3 adrenergic receptor receptor ( $\beta_3$ AR ) was cloned from a human cDNA library and expressed in Chinese Hamster Ovary (CHO) cells. The engineered CHO cells overexpressing the  $\beta_3$ AR receptor were profiled thermally for their responsiveness to the well characterized  $\beta$ -AR agonist, isoproterenol (Fig. 5). As shown in Fig. 5, CHO cells were responsive in a dose-dependent manner to isoproterenol, indicating infrared thermography can be used to evaluate, identify and rank order ligands for cell surface receptors (e.g.  $\beta_3$ AR). These results further validate the use of infrared thermography as a non-invasive tool that can be used to rank, select and identify compounds for drug discovery using engineered cell models overexpressing foreign proteins or can be

extended to antisense expression. Furthermore, infrared imaging can be used to monitor the activity of intracellular kinase activity as exemplified by the dose-dependent response of CHO cells when administered the well-characterized Protein Kinase A (PKA) agonist, forskolin (Fig. 5).

- 5 Thus, infrared thermography can be used to monitor agents that affect intracellular enzymes.

#### EXAMPLE 4

##### Infrared Analysis of Cells Treated with PPAR $\gamma$ and $\beta$ -AR Agonists: Effects on Cell Growth and Differentiation

10 It was of interest to determine if infrared thermography could be used to analyze the pharmacological effects of drugs which alter fuel metabolism. Troglitazone is an antidiabetic agent that increases  
15 anabolism (e.g., lipogenesis and mitochondrial mass) and decreases catabolism (e.g., basal lipolysis and aerobic respiration) in C3H10T1/2 cells (Lenhard et al, Biochem. Pharmacol. 54:801-808 (1997)). The effects of troglitazone on these cells is a result of activation of the transcription factor PPAR $\gamma$ /RXR which, in turn, induces differentiation of  
20 the stem cells into adipocytes (Lenhard et al, Biochem. Pharmacol. 54:801-808 (1997), Paulik and Lenhard, Cell Tissue Res. 290:79-87 (1997), Lehmann et al, J. Biol. Chem. 270:12953-12956 (1995)). Thus, infrared thermography was used to test the affects of troglitazone and 5 structurally related agonists on heat production in C3H10T1/2 cells; the  
25 effect of these agents on cellular triglyceride accumulation was also

measured as a marker for adipogenesis (Figs. 6 and 7). As shown in Fig. 6, troglitazone treatment increased triglyceride accumulation in these cells, consistent with the observation that this drug promotes adipogenesis (Lenhard et al, Biochem. Pharmacol. 54:801-808 (1997), Lehmann et al, J. Biol. Chem. 270:12953-12956 (1995)). In contrast, heat production decreased in cells treated with increasing concentrations of troglitazone or the other related PPAR $\gamma$  agonists (Fig. 7), suggesting thermogenesis is suppressed as these cells differentiate into adipocytes. Further, the rank order potencies of the various PPAR $\gamma$  agonists tested in the thermogenesis and lipogenesis assays were BRL49653 ( $EC_{50}(\mu M)=0.063$ )  $\sim$  GW1929 ( $EC_{50}(\mu M)=0.052$ ) > troglitazone ( $EC_{50}(\mu M)=0.316$ )  $\sim$  pioglitazone ( $EC_{50}(\mu M)=0.389$ ) > ciglitazone ( $EC_{50}(\mu M)=0.123$ ) > englitazone ( $EC_{50}(\mu M)=\geq 10.0$ ) (Fig. 7). Since UCP expression increases as stem cells differentiate into adipocytes (Paulik and Lenhard, Cell Tissue Res. 290:79-87 (1997), Tai et al, J. Biol. Chem. 271:29909-29914 (1996)), these observations suggest that increased UCP expression is not sufficient for stimulation of thermogenesis in adipocytes. This finding is in agreement with the suggestion that in addition to increased UCP expression other signals (e.g.,  $\beta$ -AR stimulation) are needed to stimulate thermogenesis in adipocytes (Lenhard et al, Biochem. Pharmacol. 54:801-808 (1997), Paulik and Lenhard, Cell Tissue Res. 290:79-87 (1997)). Moreover, these results indicate infrared thermography can be used to study the pharmacological effects (i.e. efficacy, potency, kinetics, etc) of agents that affect cell growth and/or differentiation, such as troglitazone and other nuclear receptor ligands, on heat production.

EXAMPLE 5

5 Intrascapular Thermogenesis and Minimum Effective Dose Correlate in  
*ob/ob* mice treated with PPAR $\gamma$  agonists

Reagents that activate the transcription factor PPAR $\gamma$ /RXR have pharmacological potential as antidiabetic agents. Infrared thermography, using the method described herein can be used to monitor the effects of such drugs in animal model systems. This application has significant importance in drug development and testing.

Mice with the genotype *ob/ob* are used as an animal model for diabetes. These animals can be used to test the activity of the antidiabetic PPAR $\gamma$  agonist GW1929x. Figure 8A shows the thermogenic effect of GW1929x treatment of a group of *ob/ob* mice. Control mice were treated in the same manner as the experimental group but with the drug vehicle lacking the drug. As expected, treatment with the antidiabetic agent for a period of two weeks prior to assay causes a reduction in IBAT thermogenesis in the treated mice compared to the control animals (Fig. 8A). This thermogenic assay in whole animals also has quantitative value for determining drug effectiveness. The minimal effective dose (MED) for a group of PPAR $\gamma$  agonists in whole animals (Henke *et al.* J. Med Chem. 41, 5020-5036 (1998)) correlates directly with the ability of the drug to suppress thermogenesis as detected by infrared thermography in cell culture (Figs. 8B and Fig. 7 described in Example 4). Thus, the rank order potency of the PPAR $\gamma$  agonists tested in cell culture plates (BRL49653 -



- GW1929 > troglitazone ~ pioglitazone > ciglitazone > englitazone)  
corresponds to the rank order of MED in animal tests ( $R^2=0.95$ ). These  
results indicate that infrared thermography can be used to study, identify,  
rank, and select compounds depending on their ability to alter  
5 thermogenesis or heat dissipation in animals.

#### EXAMPLE 6

##### $\beta_3$ -AR-Mediated Catabolism and Thermogenesis: Measuring Metabolically-Mediated Thermal Activity

- Catecholamines are postulated to regulate body temperature and  
10 composition (Blaak et al, Int. J. Obes. Relat. Metab. Disord. 17 Suppl  
3:S78-S81 (1993)), possibly by regulating UCP expression (Rehnmark et  
al, J. Biol. Chem. 265:16464-16471 (1990)) or activity. In adipocytes,  
catecholamines activate  $\beta$ -adrenoceptors ( $\beta$ -ARs) resulting in stimulation  
of the intracellular cAMP pathway (Lafontan and Berlan, J. Lipid Res.  
15 34:1057-1091 (1993)). Stimulation of the  $\beta$ -AR pathway by  
norepinephrine injection in animals mimics cold-induced increases in  
UCP1 mRNA expression in IBAT (Silva, Mol. Endocrinol. 2:706-713  
(1988)). Similarly, norepinephrine (Rehnmark et al, Exp. Cell Res.  
182:75-83 (1989)) and  $\beta_3$ -AR-agonists (Champigny and Ricquier, J. Lipid  
20 Res. 37:1907-1914 (1996)) directly stimulate UCP1 expression in cell  
culture. Thus, it is often assumed that the thermogenic effects of  
catecholamines and  $\beta_3$ -AR-agonists are mediated by increased UCP  
expression. However, it has yet to be determined if  $\beta_3$ -AR-agonists  
induce thermogenesis in the absence of increased UCP expression.

The  $\beta_3$ -AR-agonists are candidate therapeutic agents for the treatment of diabetes and obesity. Although, the mechanism of action for these agents is thought to involve increased metabolic rate (Scarpace, Ann. N.Y. Acad. Sci. 813:111-116 (1997)), it has yet to be shown that  $\beta$ -AR-agonists increase heat output by adipocytes. Therefore, it was determined whether infrared imaging could be used to monitor the effects of  $\beta$ -AR-agonists on thermogenesis in cultured adipocytes.

Thermogenesis in C3H10T1/2 adipocytes was stimulated by treatment with the selective  $\beta_3$ -AR-agonist, CL316243, and the non-selective  $\beta_3$ -AR-agonist, isoproterenol. The  $\beta_1$ -AR agonist, RO363, and  $\beta_2$ -AR-agonist, albuterol, were less effective than CL316243 or isoproterenol at stimulating thermogenesis in these cells, indicating that infrared thermography can be used to determine the selectivity and efficacy of multiple ligands. In contrast to  $\beta$ -AR-agonists, the mitochondrial electron transport inhibitor, rotenone, inhibited thermogenesis of cells treated with 50 nM of the various  $\beta$ -AR-agonists (Fig. 9). An inhibitor of protein synthesis, cycloheximide (100 $\mu$ M), had no effect on  $\beta_3$ -AR-mediated thermogenesis in these cells. Furthermore, thermogenesis was greater after 15 min than after 18 hours treatment with CL316243 (there was a dose dependent thermogenic effect of CL316243 (dose range used was 0.8-100nM)). Thus,  $\beta_3$ -AR-induced thermogenesis may be an acute response that does not require increased protein (e.g., UCP) synthesis. However, as suggested by others, these results do not preclude a role for  $\beta_3$ -AR in regulating UCP synthesis (Rehnmark et al, J. Biol. Chem. 265:16464-16471 (1990), Lafontan and Berlan, J. Lipid Res. 34:1057-1091 (1993), Silva, Mol. Endocrinol. 2:706-713 (1988)).

As a control, the effects of  $\beta$ -AR-agonists on lipolysis was also measured. Dose response analysis revealed the various  $\beta$ -AR-agonists had similar  $EC_{50}$ s in both the thermogenesis and lipolysis assays (Table 1). A correlation coefficient of 0.99 was observed when the

5 potencies of the various  $\beta$ -AR-agonists were compared in both assays suggesting the same  $\beta$ -AR signaling pathways mediate lipolysis and thermogenesis in adipocytes. Taken together, these results validate using infrared thermography to monitor ligand-altered metabolic activities in

10 cells. In general, this expands to measuring thermogenic activity of both catabolic and anabolic mediated-effects of potential drug candidates.

Table 1

Treatment	$\beta$ -AR Receptor	EC <sub>50</sub> (nM)	
		Lipolysis	Thermogenesis
Oligomycin	-	-	9.2
Rotenone	-	-	8.5
RO363	$\beta_1$	1000	956
Albuterol	$\beta_2$	47.8	43.2
Isoproterenol	$\beta_1\beta_2\beta_3$	27.1	28.0
CL316253	$\beta_3$	7.9	8.9

### EXAMPLE 8

#### Effect of Peptides on Cell Surface Receptors; Measuring Enzymatic Reactions

Vascular endothelial growth factor (VEGF) is a dimeric hormone  
5 that controls much of vascular development through binding and  
activation of its kinase domain receptor (KDR). In response to VEGF,  
cells rapidly activate a cascade of signal transduction molecules such as  
mitogen-activated protein kinases (e.g. MAPKKK, S6 kinase (p90rsk)).  
VEGF also causes phosphorylation and activation of transcription factor 2  
10 in cardiac myocytes. (Ferrara, N., Davis-Smyth T, Endocr Rev 1997  
Feb;18(1):4-25). The current available methods of analyzing this pathway  
cannot capture acute responses to VEGF. Since VEGF activation entails  
acute kinase/phosphatase activity, which ultimately entails bond  
hydrolysis and formation, heat generated from these enzymatic reactions  
15 may be measurable providing there is enough sensitivity of the instrument  
being used. To test this hypothesis, infrared thermography was used to  
measure the heat generated by human epithelial vascular cells (HUVEC)  
cells treated with VEGF. As shown in Figure 10, VEGF induced  
thermogenesis in HUVEC cells, indicating infrared thermography may be  
20 used to monitor enzymatic reactions (e.g., kinase/phosphatase activity) in  
cell culture. Thus, infrared thermography can be used to evaluate the  
efficacy and potency of compounds on enzymatic reactions.

### EXAMPLE 9

#### Monitoring Chemical Reactions (e.g. Ligand/Binding-Partner Interactions)

The formation and hydrolysis of bonds is a process intrinsic in receptors binding to their respective ligands. This process usually entails an exothermic reaction and to date has only been measured by utilizing microcalorimetry (Koenigbauer, Pharm. Res. Jun;11(6):777-783 (1994)). These measurements are the basis for establishing binding isotherms for receptor/drug interactions. Since the mixing of acid and base elicits an exothermic response generated from the process of bond hydrolysis/formation, an acid/base titration was used to determine whether or not infrared imaging could be used to measure intra/inter-molecular bond dynamics. As shown in Figure 11, the mixing of 0.25 mM NaOH with varying concentrations of HCl exhibited a dose-dependent thermogenic response when measured by infrared thermography. These data validate the utility of infrared thermography in measuring molecular events such as ligand (e.g., drug) interactions with binding partners (e.g., receptors). Further examples include using infrared thermography to monitor drug-receptor, protein-protein, protein-DNA, DNA-DNA, DNA-RNA and protein-carbohydrate interactions.

### EXAMPLE 10

#### Monitoring Catalysis on Inert Solid Surfaces (e.g. Combi-Chem beads)

Infrared thermography as presented in this invention can also be used to measure thermogenesis in highly defined cell-free systems.

Catalytic agents are often characterized while immobilized by attachment to inert solid surfaces such as combi-chem beads (Borman, Chem. Eng. News 74:37 (1996)). Thermal analysis of catalyst reactions on combi-chem beads was therefore tested using the present invention.

- 5 Catalytically active combi-chem beads were analyzed while immersed in solvent in a 25 mL beaker or in a 96 well microtiter plate. Fig 12 shows that thermogenic output localized to the area of the beaker containing the beads (Fig. 12A, arrows) (0.3°C temperature difference) or to the wells of the microtiter plate that contain active but not inactive beads (Fig. 12B).
- 10 Thus, infrared thermography can be applied to measure real-time catalyst activity in a non- invasive and non-destructive manner.

#### EXAMPLE 11

##### 15 Thermographic Analysis of Aerosol-Induced Cooling

- Aerosol systems, metered dose inhalers (MDI), used in drug delivery are associated with decreased temperature in the apparatus chamber during delivery. This effect has significant implications for the efficiency of drug delivery. A decrease in chamber temperature often causes drug crystallization within the MDI chamber and stem, ultimately, resulting in inefficient delivery of the drug. Infrared thermal imaging can be used to monitor the temperature loss during drug delivery and to test modified devices that alleviate or ameliorate the problem. Thus, infrared
- 20 thermography was tested as a measure of actuation-induced chamber cooling of a MDI. Figure 13A shows a thermal profile in real time of an MDI after 0,1 or 5 consecutive actuations. The thermal image was
- 25

analyzed for temperature fluctuation in the three following areas: area 1 - the surface of valve stem/expansion chamber; area 2- the surface of the middle of the canister; area 3 - the canister head inside which sits the metering chamber. The graphical representation of area temperature over time shown in Fig. 13B indicates that an actuation-dependent temperature decrease occurs specifically in the valve stem/expansion chamber. Iterative drops in temperature occur with each actuation.

Furthermore, the bioavailability and bioactivity of inhalants in animals and man is often difficult to measure and quantitate. Assays available for measuring bioavailability of inhalants involve measuring the uptake of radiolabeled inhalant in selected tissues (eg. lung). Infrared thermography provides a non-invasive method for measuring the bioavailability/bioactivity of inhalant compounds. Thus, infrared thermography was used to measure the thermal activity induced by an inhalant in the thoracic area of nude mice. Figure 13 C shows a thermal profile of nude mice that were treated with inhalants containing either vehicle or albuterol. The graphical representation of the torso area temperature indicates that the torso temperature increases after 2.5 minutes of dosing with inhalant (Fig. 13D). Taken together, these results indicate that infrared thermography can be used to non-invasively measure the delivery of inhalants in the devices as well as the bioavailability/bioactivity of inhalants in animal models.

EXAMPLE 12

Infrared Thermography Can Be Used to Measure IBAT Thermogenesis in  
Mice Treated with  $\beta_3$ -AR-Agonists

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The ability of  $\beta_3$ AR-agonists to stimulate thermogenesis in cultured adipocytes and CHO cells was shown and discussed in Examples 3 and 6 and Figures 5, 8 and 9 above. Since there are potential clinical applications for the use of catabolic agents (e.g.,  $\beta_3$ AR-agonists) in the treatment of diabetes and obesity, it is important to show that infrared thermography can measure  $\beta_3$ AR-agonist-induced effects in whole animals. Figs. 14A and 14B show that infrared thermography can be used to measure a dose-dependent and time-dependent increase in the intrascapular brown adipose tissue region (IBAT) thermogenesis in animals challenged with a  $\beta_3$ AR-agonist. The hypothesis that  $\beta_3$ AR agonist-induced thermogenesis reflects increased catabolic activity was tested by direct measurement of serum glycerol in treated animals.  $\beta_3$ AR agonist-induced thermogenesis correlates with increased serum glycerol in treated mice (correlation coefficient = 0.92). Thus, this validates the use of infrared thermography to monitor the *in vivo* effects of catabolic agents in animals.



### EXAMPLE 13

#### Thermographic Analysis of *ob/ob* Mice Treated with a Monoamine Reuptake Inhibitor Over Various Time Points and Drug Concentrations

Monoamine reuptake inhibitors are a class of drugs that stimulate catabolic activity (Stock, Int. J. Obesity 21:525-29 (1997)). The effect of a representative monoamine reuptake inhibitor, GW473559A was monitored by infrared thermography of treated mice. Figure 15 shows that infrared thermography measures a dose-dependent (Fig 15A) and a time-dependent (Fig. 15B) increase in IBAT thermogenesis in *ob/ob* mice treated with GW473559A. Thus, infrared thermography provides a non-invasive, sensitive, and robust surrogate assay for the bioavailability and activity of compounds.

### EXAMPLE 14

#### Thermographic Analysis as a Surrogate Assay to Predict Body Weight Changes

Treatment with  $\beta_3$ AR-agonists to control diabetes or obesity can require therapy that spans extended time periods (weeks or months). One desired outcome of  $\beta_3$ AR-agonist treatment can be weight loss. Data shown in Figure 16 demonstrate that infrared thermography can be used to predict weight loss resulting from drug treatment. AKR mice were

placed on a high fat diet and treated with either placebo or  $\beta_3$ AR agonist (twice daily) for 2 weeks. During the study both thermogenic activity as measured by infrared thermography of the intrascapular area and weight loss were measured. Weight loss after two weeks correlated with the intrascapular area thermogenesis ( $r=0.97$ ). Thus, infrared thermography can provide a non-invasive surrogate assay for both preclinical and clinical use for compound selection, and evaluation of efficacy and potency.

Drug treatments for weight reduction in diabetic and obese patients is often a long-term therapy. Infrared thermography detects the drug induced metabolic change whenever the drug is administered. Changes in IBAT thermogenesis recorded by infrared thermography after animals were treated with monoamine reuptake inhibitors provide a valid correlation for % reduction in weight gain for both acute (treatment = 2 hrs;  $r = 0.92$ ) and chronic (treatment = 14 days, 2 hrs post-dosing;  $r = 0.94$ ) treatment protocols. Infrared thermography provides a non-invasive surrogate assay for predicting weight loss, thus, precluding labor intensive, long-term and expensive weight loss studies.

#### EXAMPLE 15

##### Genetic Influence on Diet-Induced Thermogenesis

Considerable evidence indicates that metabolism is influenced by genetic and environmental factors. One environmental component that alters the metabolic rate in individuals with a genetic predisposition

towards diabetes, dyslipidemia, or obesity is diet. It was unknown if thermography could be used to measure the effects of diet on drug-induced changes in heat production in animals with different genotypes.

Thus, three different inbred strains of mice, AKR/J, C57BL/6J, and

- 5 SWR/J, were maintained on high and low fat diets for 14 weeks before treating with the  $\beta_3$ -adrenoceptor agonist, BRL37344. The heat produced in the intrascapular region was measured before and after 60 min treatment using infrared thermography. As shown in Figure 17, the obesity prone mice, AKR/J, had a greater thermogenic response to
- 10 BRL37344 when fed the higher fat diet. In contrast, the obesity resistant mice, SWR/J, had a greater thermogenic response when fed the lower fat diet. There was little difference in the thermogenic response of C57BL/6J mice on a high or low fat diet. These results validate using infrared thermography to measure how genotype and environmental changes
- 15 (e.g., diet) effect drug-induced changes in heat output. Thus, this technique may be used to select, rank order for the efficacy and potency of compounds with the desired properties for a given environment or genetic background. Similarly, this technique can be used to identify, rank, and select animals and environmental factors with the desired
- 20 properties for a given drug candidate.

### EXAMPLE 16

#### Infrared Thermography of Diet-Induced Thermogenesis in Humans

5           Caloric uptake has a dramatic and acute influence on metabolic activity in humans. Thus, the thermogenic output of the dorsal area of a human subject will vary as a function of the time of day and the subject's pattern of food intake. This is demonstrated in a profile of a patient whose dorsal temperature is monitored by infrared thermography at time points  
10 before and after a meal. Figure 18A shows quantitative analysis of the thermographic profile for time points before and after lunch. Figure 18B shows a graph summarizing similar measurements made in 2 male subjects and 1 female subject before and after lunch (Torso Delta T) on three separate occasions. This data set is consistent and reproducible,  
15 and indicates that infrared thermography of humans is possible for monitoring thermogenesis. This method may be useful to monitor many situations applicable to human patients, such as diet modulation, drug treatment, drug/drug and drug/environment interactions. Based on the results using infrared thermography, changes in diet and environment and  
20 drug use can be prescribed.

### EXAMPLE 17

#### Infrared Thermography of Drug-Induced Thermogenesis in Humans

25

The sympathomimetic agent ephedrine has been reported to have potent thermogenic and anti-obesity properties in rodents (Astrup et al,

Am. J. Clin. Nutr. 42:183-94 (1985)). Weight loss and body composition measurements are markers primarily used to determine the efficacy of pharmacological treatments for obesity. However, studies utilizing these markers tend to be time consuming, large and costly. In order to  
5 circumvent these problems, surrogate markers have been developed. Indirect calorimetry is used to determine resting metabolic rate but due to its complexity, it is not widely used. Biochemical markers such as glucose, glycerol, nonesterified fatty acids, triglycerides have been used but are invasive. However, thermogenic imaging has never been used to  
10 measure the properties of ephedrine in humans.

The effect of ephedrine on thermogenesis in two human subjects was detected by infrared imaging 60 minutes after treatment with ephedrine at a dose of 0.6-0.7 mg/kg. Figure 19 is a thermal image demonstrating the thermogenic response induced by ephedrine in  
15 subjects A and B ( $\Delta T^{\circ}\text{C} = 0.63$  and  $0.46$ , respectively). Thus, infrared thermography can be used as a non-invasive surrogate assay to evaluate the efficacy, potency, pharmacokinetics, pharmacodynamics of drugs in clinical studies.

## 20 EXAMPLE 18

### Infrared Thermography of Drug-Drug Interactions in *db/db* Mice

The outcomes of pharmacodynamic drug-drug interactions are  
25 diverse and may range from potentially life-threatening situations to life saving therapies. As such, there is a great need to identify methods for rapid evaluation of drug interactions. Given a number of drugs alter

metabolic rate, it was postulated there may be additive, subtractive, and/or synergistic effects of various agents on heat production in cell culture and/or in animals. Thus, it was of interest to determine if infrared thermography could be used to analyze the effects of mixing different agents on thermogenesis *in vivo*. Again, infrared thermography can be used to identify, rank order the efficacy, potency and toxicity of combination therapies in both pre-clinical and clinical studies.

GW1929 is an agent that improves glycemic control in diabetic animals by activating transcriptional activity of the ligand-activated nuclear receptor PPAR $\gamma$ . CGP12177A is an anti-obesity agent that acts via stimulation of the cell-surface  $\beta_3$ -adrenoceptor (Kenakin, Lenhard and Paulik, Curr Prot Pharm; 1(unit 4.6):1-36 (1998)). Since many diabetic patients are obese, it was of interest to determine if these two agents (*i.e.*, GW1929 and CGP12177A) had any pharmacodynamic interactions.

Thus, *db/db* mice were treated for 2 weeks with or without GW1929. At the end of the study, the animals were treated with or without CGP12177A and the heat produced in the intrascapular region was measured using infrared thermography. As shown in Figure 20, there was a greater thermogenic response in animals treated with both GW1929 and CGP12177A than either agent alone. Thus, these results validate using infrared thermography to measure how pharmacodynamic drug-drug interactions alter heat production.

### EXAMPLE 19

#### Thermographic Analysis of Tissue Vascularization by VEGF

5 Tumors are actively growing and metabolizing regions of tissue supported by local vascularization that enhances the tumor growth potential. Thermography can detect tumor-associated increases in thermogenesis, including those associated with tissue vascularization. Vascular endothelial growth factor (VEGF) is found in tumor tissue and its  
10 presence is associated with increased thermogenesis in the tissue in which it is localized. Thus, a nude mouse model was used that has its epidermal vasculature exposed to demonstrate the usefulness of thermography in tumor detection and vascularization. Nude mice were injected with either VEGF peptide or a control followed by themographic  
15 imaging of both injection sites. Figure 21 shows thermographic images that demonstrate enhanced thermogenesis in the local area of the VEGF injection, but not in the local area of the control injection. Thus, infrared imaging can be used to monitor the effects of agents that alter tissue vascularization.

20

### EXAMPLE 20

#### Monitoring Tumor Temperature by Infrared Thermography

25 Tumor temperature is an indicator of the metabolic rate in the tumor. Tumors are dependent on the presence of VEGF for maximum metabolic activity and tumor temperature reflects changes in the

availability of VEGF. This relationship is demonstrated by thermographic analysis of tumor-bearing mice treated with either an anti-VEGF antibody or a nonspecific anti-IgG antibody. Figure 22 shows quantitative thermographic analysis demonstrating that tumor temperature decreases  
5 when VEGF is neutralized by the presence of anti-VEGF antibody. Thus, infrared imaging can be used to monitor the effects of anti-cancer therapies and as an aid in anti-cancer drug development.

#### EXAMPLE 21

10

##### Monitoring Alopecia (Hair-loss) by Infrared Thermography

Hair loss can result from undesirable side-effects of various therapies (e.g., radiation treatment of cancer patients, surgery etc.) and  
15 can occur naturally with age, whereas surgical or pharmacological intervention can restore hair growth. Since hair provides insulation against heat loss and aids in the maintenance of a constant body temperature, it was of interest to determine if infrared thermography can be used to measure hair loss. The lack of progress in the treatment and  
20 prevention of chemotherapy-induced alopecia is in part due to the lack of a reproducible animal model as well as a quantitative method to measure hair-loss. To determine if infrared imaging can be used to quantitatively measure chemotherapy induced hair-loss, newborn rat pups that were treated in utero with the known cancer agent, etoposide, were thermally  
25 profiled for hair-loss. Fig. 23 shows thermal images (Fig. 23A) and quantitative analysis (Fig. 23B) demonstrating increased thermal activity in both the fronts and backs as a result of hair-loss. Thus, these results



validate using infrared thermography to measure hair-loss as a method to aid in identifying therapies for alopecia and hair-loss in general or to identify agents that can cause hair-loss as an adverse reaction (e.g. a toxicity screen).

5

#### EXAMPLE 22

##### Thermography After Drug Treatment for Male Erectile Dysfunction

10 Male erectile dysfunction (MED) is treatable with drugs that increase blood flow to the genitalia. Increased local thermogenesis is associated with increased local blood flow. One drug that acts in this manner and treats MED is Pinacidil. Figure 24 shows that infrared thermography detects a Pinacidil-induced increase in thermogenesis in  
15 the genitalia of rats 2 hours after dosing with either 3.0 or 0.3 mg Pinacidil /kg. Thus, infrared thermography provides a quantitative and non-invasive method for identifying and evaluating drug candidates for the MED indication as well as identifying candidates that can cause erection as a side effect.

20

#### EXAMPLE 23

##### Monitoring the Effect of Antiinflammatory Agents by Thermography

25 Arthritis is a disease characterized by inflammation of the joints and can be treated with antiinflammatory agents. Because inflammatory responses are associated with increased thermogenesis at the site of the

response, arthritis and the efficacy of arthritis drugs can be monitored by thermography. To demonstrate this application, an arthritis model was established by injecting one limb of a normal animal with peptidoglycan polysaccharide (PGPS) for two weeks. The other limb was not treated.

- 5 Infrared thermography on both limbs after the arthritis-inducing treatment demonstrates a higher level of thermogenesis in the treated limb than in the untreated limb (Figure 25A) indicating infrared thermography can be used to monitor the ability of agents to cause inflammation. Subsequent treatment with the antiinflammatory agent prednisolone reverses the
- 10 elevation in thermogenesis, such that both limbs have similar thermographic profiles after treatment (Fig. 25B). Thus, infrared thermography is a useful tool for monitoring inflammatory responses and the efficacy of antiinflammatory agents as well as providing a method for screening, selecting and evaluating the effectiveness of drug candidates
- 15 for treating arthritic indications.

All documents cited above are hereby incorporated in their entirety by reference.

- One skilled in the art will appreciate from a reading of this
- 20 disclosure that various changes in form and detail can be made without departing from the true scope of the invention.